

Bringing methanotrophy in rivers out of the shadows

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Abstract

Methane oxidation produces biomass that is a potential source of particulate carbon for consumers, and is in addition to photosynthetic production. We assessed methanotrophy and photosynthetic production under differing conditions of light and methane concentration. We measured methane oxidation and photosynthesis in gravel sediments from adjacent shaded and unshaded stretches of 15 chalk rivers in southern England, and also in 30 artificial channels in which we manipulated light and methane experimentally. The capacity for methane oxidation was 78% higher in the shade than unshaded areas, indicating a denser, or more active, methanotrophic assemblage on shaded riverbeds, and the difference was most pronounced when methane concentration was high. Across the 15 rivers, methanotrophic production ranged from 16 to 650 nmol C cm⁻² d⁻¹ and net photosynthetic production from 256 to 35,750 nmol C cm⁻² d⁻¹. The relative importance of methanotrophy to their total production (i.e., photosynthetic and methanotrophic) increased with methane concentration and ranged from 0.1–2.4% and 0.2–13% in unshaded and shaded areas, respectively. Over an annual cycle in one river, the response of the methanotrophs in the shade to a high summer methane concentration was ~ five times greater than in the open; in winter, there was no effect of shading on methane oxidation. The response of methanotrophy to shading and methane concentration in the artificial channels resembled that found in the rivers. Methanotrophy makes a non-negligible (here up to ~ 13%) contribution to particulate carbon production in these streams, is disproportionately greater in the shade, and constitutes a distinct carbon pathway available for their food webs.

Some recent efforts to integrate fresh waters into the global carbon cycle have focussed on understanding the role of methane (Bastviken et al. 2011; Yvon-Durocher et al. 2014; Segarra et al. 2015; Holgersson and Raymond 2016). Good progress has been made in estimating methane flux from freshwaters to the atmosphere (Striegl et al. 2012; Melack et al. 2013; Sawakuchi et al. 2014; Stanley et al. 2016), but the methane cycling within the riverbed and how this might change the balance of basal resources available to the food web is still unclear.

Methanotrophic bacteria use methane as their sole carbon and energy source (Hanson and Hanson 1996); the fraction that is not assimilated is oxidized to carbon dioxide, a less

potent greenhouse gas. While methanotrophs are not autotrophs, their ability to synthesize biomass from methane enables this gas to be converted into a form available to animals, and it would otherwise be lost from the ecosystem. Microbial methane oxidation has been well studied in lakes and wetland sediments, where the methane concentration can be high (μ M–mM range) (Hershey et al. 2015; Oswald et al. 2015; Segarra et al. 2015) and methanotrophic production contributes considerable particulate carbon to the food web to the extent that it can be detected in the biomass of macroinvertebrates and fish, and even exported to the terrestrial ecosystem via emerging adult insects (Grey and Deines 2005; Ravinet et al. 2010; Jones and Grey 2011; Grey 2016).

In low methane environments (nM concentrations), particularly where photosynthetic production is high, very little attention has been paid to the potential significance of methane and methanotrophic production. However, we recently showed that production derived from methanotrophy can rival photosynthetic production in rivers where the bed-sediments are well irrigated with methane and oxygen below the photic zone (Shelley et al. 2014). Given the wide

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reporting of high methane concentrations in rivers from a range of latitudes (Belger et al. 2011; Ortiz-Llorente and Alvarez-Cobelas 2012; Sawakuchi et al. 2014; Stanley et al. 2016) we need to explore methane dynamics further in these systems, which are well recognized as biogeochemical “hotspots” in the landscape (Aufdenkampe et al. 2011).

The concentration of methane is a key factor determining its rate of oxidation (Duc et al. 2010; Shelley et al. 2015). In lakes, and especially those that stratify, methane production follows a seasonal pattern, reaching a maximum in the sediments in late summer, related to anoxia (Deines et al. 2007). In many rivers, the methane concentration in the water column is also seasonal, with a low baseline derived from groundwater over winter and a summer peak due to augmentation by methanogenesis in patches of anoxic fine sediments [in some cases at least, the organic matter originating from farmland (Walling and Amos 1999) trapped in and around macrophyte stands (Sanders et al. 2007)]. For example, in the River Frome in SW England, there was a sevenfold increase from the winter minimum to an August peak of $1.43 \mu\text{mol CH}_4 \text{ L}^{-1}$ (Sanders et al. 2007), while in the River Lambourn there was a more modest doubling of methane concentration from approximately $100 \text{ nmol CH}_4 \text{ L}^{-1}$ to over $200 \text{ CH}_4 \text{ nmol L}^{-1}$ (Trimmer et al. 2009). At the concentrations encountered in English chalk rivers (Shelley et al. 2014), we have found that methane oxidation is strongly substrate limited and so the rate of its oxidation in situ increases proportionally with the change in methane concentration through the summer (Shelley et al. 2014). Furthermore, the biomass of methanotrophs is greater when the ambient methane concentration is higher (Bender and Conrad 1995; Deines et al. 2007; He et al. 2012), although we are not aware of any published relationships between methane concentration and methane oxidation capacity across different rivers. We use the phrase “methane oxidation capacity” to describe differences in methane oxidation rates when incubated with the same initial concentration of methane i.e., removing any kinetic effect.

Intense land-use change and the direct management of rivers has perturbed biogeochemical cycling in riverbeds (Crawford and Stanley 2016; Sieczko et al. 2016). Notably, fine sediment pollution in agricultural catchments has led to increases in river methane concentrations (Sanders et al. 2007; Crawford et al. 2014) and reductions in riparian shading (through increased light and decreased detrital input) can modify the sources of energy supporting food webs (Wootton 2012). Current river management practices around the globe involve restoring, rehabilitating, or protecting riparian vegetation to create a buffer strip of native species that will help to mitigate against soil erosion, nutrient pollution, and physical degradation via livestock trampling (Clews et al. 2010; Doskey et al. 2010; Pander and Geist 2010). Denser riparian shading can be used as a management tool to control nuisance algal blooms, maintain or reduce water temperature, and

overall to return rivers to good ecological status, particularly in systems where nutrient concentrations are high (Rosemond et al. 2000; e.g., Hutchins et al. 2010; Sturt et al. 2011; Bowes et al. 2012; Johnson and Wilby 2015). The overhanging canopy reduces photosynthetically active radiation (PAR) reaching the water surface, sufficient to reduce benthic photosynthesis (Lamberti et al. 1989; Hill et al. 1995; Julian et al. 2011). Thus, even if methanotrophy is not directly affected by light, reduced photosynthesis in shaded reaches would make methanotrophy a relatively more important carbon source for the food web compared with unshaded reaches.

Alternatively, methane oxidation may be directly affected by light, although the evidence in the literature is contradictory and does not easily translate to riverbed systems. High light inhibited methane oxidation in the surface waters of some lakes (Dumestre et al. 1999; Murase and Sugimoto 2005), although in lake-water bottle incubations there is likely to be a strong interaction with photosynthesis whereby in the light, carbon dioxide is quickly removed pushing the pH above 9, which inhibits methanotrophy. Whether this light-inhibition is direct, as has been widely observed in ammonia-oxidizing bacteria (which are structurally similar to methanotrophs) (Merbt et al. 2012), or indirect—associated with photosynthesis—is still unknown. Elsewhere, in a wetland, light was shown indirectly to enhance methane oxidation, because photosynthesis caused a vertical expansion in the oxic surface layer thus increasing the zone suitable for methane oxidation (King 1990). There is a clear knowledge gap with regards to the direct and indirect effect of light on methane oxidation in general, and a complete absence of previous attempts to understand this in riverbed sediments.

Here, we exploited the wide natural variation found in riverbed shading and methane concentration across streams in southern England, as a means of testing the effect of these two factors on the relative importance (in terms of carbon gases assimilated into biomass) of methanotrophy and photosynthesis for the production of particulate carbon in lowland rivers. Further, as an initial assessment of seasonal changes, we sampled one river a number of times over an annual cycle during which shading and the dissolved methane concentration changed markedly. Finally, we constructed a set of experimental channels fed from spring water to isolate the response of gravel biofilms to controlled manipulations of methane and light.

We hypothesized that methanotrophic production would be positively correlated with ambient methane concentration and thus that methane-derived carbon would be relatively more important in methane-rich rivers. We expected that, since shading limits benthic photosynthesis, methanotrophy would be proportionally more important (relative to net photosynthetic production) in shaded reaches, even if the methanotrophs are unaffected by light. Finally, our experimental design allowed us to test whether shading and methane affected the capacity for methane oxidation.

Table 1. Site details. Water temperature and pH were measured at mid-channel and mid-depth and mean concentrations for water gases and chemistry are reported. Surface irradiance [Light (% difference)] is reported as the percent reduction in the shade compared to unshaded stretches at each river and are means of at least 20 measurements. Nutrient concentrations are means of three replicate water samples, filtered, and frozen on site, measured on a Skalar San++ continuous flow analyser in the laboratory. *ambient methane concentration.

River	Date sampled	Order (upstream section)	Water temperature (°C)	pH	Light (% dif)	ΣDIC (mM)	CH ₄ (nM)	pCO ₂ (μM)	Nitrate (μM)	Nitrite (μM)	Ammonium (μM)	SRP (μM)
Misbourne	24 Aug 11	Shaded	18.5	8.26	68	4.2	38	117	866	1.7	10.1	4.2
Chess	24 Aug 11	Shaded	16.5	8.19	80	4.2	42	148	785	4.1	13.1	5.4
Itchen	15 Aug 11	Unshaded	14.5	7.84	92	4.1	49	274	688	2.3	6.5	0.5
Cray	20 Aug 11	Shaded	20	8.15	98	3.8	51	512	245	2.2	7.1	0.6
Allen	29 Aug 11	Unshaded	16.3	8.21	89	3.7	63	117	994	0.8	3.5	0.2
Granta	23 Aug 11	Shaded	17.5	8.11	96	3.8	66	150	1221	2.4	15.6	36.4
Test	15 Aug 11	Unshaded	15.3	7.99	92	3.9	71	83	774	1.2	6.1	0.4
Lambourn	16 Aug 11	Unshaded	14.5	8.3	91	4.2	71	120	923	1.4	3.5	1
Meon	29 Aug 11	Shade	14	8.75	91	3.7	74	102	732	0.9	4.4	0.4
Stort	23 Aug 11	Unshaded	15.6	7.8	90	3.1	90	286	1716	1.9	10.3	82.4
Darenth	20 Aug 11	Shaded	17.8	8.1	74	2.5	94	346	196	0.7	4	0.5
Bourne	16 Aug 11	Unshaded	16.3	8.14	88	3.7	111	131	880	1.3	5.5	1.3
Frome	30 Aug 11	Shaded	14.4	8.27	93	4.1	147	151	407	0.7	4.3	1.4
Bere	29 Aug 11	Unshaded	16.2	8.11	95	4.2	192	202	946	1.8	5.5	0.5
Piddle	30 Aug 11	Unshaded	14.3	8.12	97	4.6	224	219	835	1.1	8.5	0.3
Fobdown	08 May 12	Experiment	10.5	7.2	66	7.7	9.43*	463	763	0.2	5.1	0.6

Methods

Survey sites

Based on an earlier extensive survey (Shelley et al. 2014), we chose sites with adjacent shaded and unshaded stretches, beds of clean gravel and relatively fast flow, all of which are typical for “healthy” chalk streams. These conditions were important, as we did not wish our results to reflect the effects of anthropogenic degradation, such as heavy siltation. Fifteen rivers fitting this description (Table 1 and *see* electronic Supporting Information S1) were surveyed in August 2011, when maximum riparian shading occurs and coincides with the peak in river-water methane (Shelley et al. 2014). At each site, adjacent shaded and unshaded stretches of river (~30 m long and normally within 100 m of each other) were identified; the one exception was the Granta, where the shaded area was ~300 m upstream of the unshaded area due to access constraints. In all cases, shading was by native riparian deciduous vegetation. One of the 15 sites, the Bere Stream, was selected for study over an annual cycle by sampling on a further six occasions. This choice was on the basis of good access, background data, and its designation (by local conservation agencies) as a “Site of Special Scientific Interest” as a typical permanent chalk stream which is largely unmanaged.

Experimental set-up

A suite of artificial channels was constructed to assess, in a controlled experiment, the effect of light and methane

concentration on the extent of methanotrophy. The experiment was located on a watercress (*Nasturtium officinale*) bed at Fobdown Farm (51.102056°N, -1.186837°W), Hampshire, UK and consisted of 30 channels (0.15 m × 0.15 m × 1.5 m; width × depth × length) arranged in five blocks of six, which were filled with a 5 cm layer of substratum (washed gravel of median particle size 20 mm), similar to that described by Harris et al. (2007) as typical of the bed of a chalk stream. Water (maintained at 2–3 cm depth) entered the channels through individual pipes (with flow control valves) from the main channel supplying the watercress bed, which was fed by a natural chalk spring (*see* electronic Supporting Information S2). Three methane treatments (low, medium, and high) and two light treatments (shaded and unshaded) were applied. Target methane concentrations were based on the summer maximum (“high methane”) and baseline winter concentrations (“medium methane”) measured in the Bere Stream (Fig. 1b). The ambient methane concentration in the groundwater at Fobdown, which was three times above atmospheric equilibration (at 10°C) on average, formed a third, “low” methane treatment. The 20 channels requiring increased (medium and high) methane concentrations had a second inflow pipe delivering water (supersaturated in methane) for which the flow was adjusted so that target methane concentrations were reached in the mid-section of each channel, while achieving similar flow rates across all channels. Methane was introduced into this

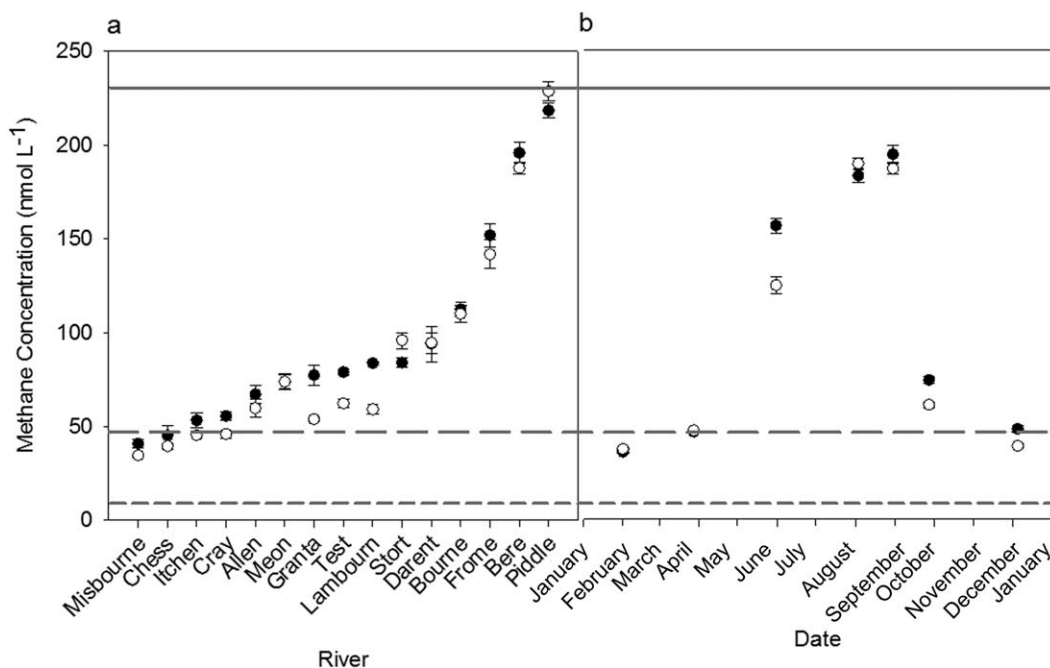


Fig. 1. Dissolved methane concentrations (mean \pm SE) in the shaded (filled circles) and unshaded (open circles) areas of (a) 15 rivers in August 2011 and (b) the Bere Stream throughout an annual cycle. Horizontal gray lines show the methane concentration achieved in the low (short dash), medium (long dash) and high (solid gray line) artificial channel treatments.

water source in a tall 400 L sealed header tank, through 16 fine bubble diffusers located at the base of the tank, from a gas cylinder containing 1.75% methane in air (BOC). All channels were covered with ultra-fine insect mesh (Harrod Horticultural) mimicking the effect of light attenuation by the water column in natural streams (Trimmer et al. 2010), and the shaded channels were covered with an additional layer of shading material (75% sun shade cloth, Bouillon S.A.R.L.). The experimental treatments were applied to the channels for 5 months, from the initial introduction of the substratum in December 2011 to the beginning of May 2012.

Dissolved CH₄, CO₂, Σ DIC, and inorganic nutrient concentrations

Dissolved CH₄ and CO₂ in the river water was quantified by taking water samples ($n = 10$ for each river section and $n = 6$ per artificial channel), at mid-depth and mid-channel using Tygon tubing attached to a 60 mL gas-tight syringe. Water was gently discharged into the bottom of a 12 mL gas-tight vial (Exetainer, Labco) and allowed to overflow (~ three times) before half were fixed (100 μ L of the bactericide ZnCl₂ 50% w/v) and all were then sealed. The samples for CO₂ analysis could not be fixed as the bactericide also acidifies the sample and converts carbonate and bicarbonate to carbon dioxide; however, in trial runs we found no change in CO₂ within the first 24 h if the vial is kept refrigerated. Upon return to the laboratory (< 24 h from collection), a 2 mL headspace (analytical grade helium, BOC) was

introduced and, following equilibration, 75 μ L from the headspace was injected into a gas chromatograph equipped with a flame ionizing detector and a hot-nickel catalyst to reduce the CO₂ to CH₄ (Agilent Technologies; Sanders et al. 2007). The concentration of CH₄ or CO₂ in the headspace was calculated from peak areas calibrated against a certified standard gas mixture (100 ppm CH₄, 2700 ppm CO₂, balance N₂, Scientific and Technical Gases) included with every run, and the amount in the original river water sample was calculated using solubility coefficients (Weiss 1974; Yamamoto et al. 1976). Subsequently, to measure total dissolved inorganic carbon (Σ DIC), 100 μ L of HCl (12.2 M) was injected into the samples through the septa to ensure complete acidification, and the concentration of CO₂ in the headspace was measured as above against a prepared inorganic carbon calibration series (0–10 mM) of sodium carbonate. This was done on fixed samples within 1 week of collection.

Inorganic nutrients (nitrate, nitrite, ammonium, and soluble reactive phosphorus (SRP)) were measured on filtered (0.45 μ m) water samples using a segmented flow auto-analyser (Skalar San⁺⁺, Breda) and standard colorimetric techniques (Kirkwood 1996).

Methane oxidation: comparable capacity and predicted in situ rates

Methane oxidation is known to be substrate limited in chalk riverbed gravels (Shelley et al. 2014, 2015) and, therefore, the amount of methane added to the incubation vial will affect the rate measured. To account for this, all

incubations began with the same methane concentration (100 nmol CH₄ L⁻¹) and we termed these comparable rates the “capacity for methane oxidation.” This is a useful metric for quantifying the true variation in the capacity of the gravel bed to oxidize methane, irrespective of changing ambient methane concentration i.e., it is a proxy for active methanotrophic biomass. On the other hand, the predicted rate of methane oxidation considers the kinetic effect of the ambient methane concentration in each river. This can be calculated from the known kinetic response to increased methane concentration.

To quantify the capacity for methane oxidation ~ 1 g of riverbed sediment ($n = 6$ for each river section, $n = 3$ per artificial channel) and 5 mL of the corresponding river water (or watercress farm spring water) were added to gas-tight vials (12 mL, as above). After sealing the vials, the air headspace was enriched with methane to give 100 nmol CH₄ L⁻¹ in the water after equilibration. Control vials were set up to test for any methane oxidation in the river water itself. The concentration of methane in the headspace of each vial was measured immediately after spiking and then at 24 h intervals for 4–5 d, in between which the samples were incubated on a rotary shaker (Stuart Scientific platform shaker STR6 set at 10 rev/min), at 11°C (average groundwater-fed river temperature in UK), in the dark. The capacity for methane oxidation was calculated using linear regression of nmol CH₄ consumed per hour during the linear phase of the incubation (72 h) and then normalized for dry mass of sediment. Using this measured capacity for methane oxidation (C_{mo} , nmol CH₄ g⁻¹ dry sediment h⁻¹) a site-specific rate (i.e., predicted rate in situ, taking account of ambient concentration at that site) was calculated using well estimated linear relationships (typically $R^2 = 0.96$, error on slope 4%) which holds well beyond the range of adjustment applied here (Shelley et al. 2014, 2015; Trimmer et al. 2015):

$$\text{Predicted rate of methane oxidation in situ } (R_{mo}) = \left(\frac{C_{mo}}{C_i} \right) \times C_{amb} \quad (1)$$

Where C_{mo} is the measured rate of methane oxidation (at 11°C in the dark), C_i is the initial methane concentration, and C_{amb} is the ambient methane concentration at the site.

Methanotrophic production

Methanotrophs synthesize biomass from methane, and in doing so they recycle carbon back into a form available to higher trophic levels. To quantify this recycling of carbon we calculated methanotrophic carbon production. Net daily methanotrophic production per cm² of riverbed was estimated using the following equation:

$$\begin{aligned} \text{Methanotrophic production (nmol cm}^{-2}\text{d}^{-1}) \\ = R_{mo} \times V \times CCE \times d \times h \end{aligned} \quad (2)$$

Whereby, R_{mo} is the rate of methane oxidation (nmol g⁻¹ h⁻¹), V is the volume in cm³ taken up by 1 g of gravel (0.95), CCE is the carbon conversion efficiency which we have shown previously to be 0.5 (50%) for eight typical chalk streams (see Trimmer et al. 2015), d is the depth over which we have integrated the methane oxidation [15 cm is the conservative estimate of riverbed depth over which methane oxidation occurs at a similar rate to that at the surface (Shelley et al. 2014)]; and h is the number of hours (per day) over which methanotrophy was assumed to occur (24). For the artificial channels experiment, d was set at 5 cm as this was the depth of gravel in the channels. We assumed continual methanotrophy (i.e., 24 h per day) in the gravels, as our laboratory experiments were performed over several days, and during this time methane was consumed linearly. Additionally, it should be noted that any instantaneous reduction in methanotrophy under high light (for which we currently have no evidence), would only impact the very surface layer of the riverbed.

Quantifying riverbed irradiance

Photosynthetically active radiation (PAR; 400–700 nm) was measured (as $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) using a Skye Quantum sensor (Skye Instruments Limited) at the riverbed, every metre along three cross-river transects ($n = 18$ –36 per river depending on channel width) which were spaced by approximately 10 m and then averaged to give mean riverbed light for each section. Shaded and unshaded sections were measured within a 10 min time frame, to minimize the effect of changing light conditions.

Photosynthesis and chlorophyll

Net photosynthesis and dark respiration were measured by logging oxygen evolution over timed light-dark gravel incubations. Gravel samples (~ 30 g, $n = 6$ for each river section and $n = 3$ for each channel) were incubated for 45 min under a light source, followed by a further 45 min in the dark inside 250 mL gas-tight Perspex incubation chambers fitted with a stirrer and oxygen electrode (OX50, Unisense) (see Trimmer et al. 2010 for further details). A photon flux density of 55 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ was generated at the gravel surface and this was reduced to 18 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for the shaded experimental channels, which were covered in cloth during incubations (as used at the experimental site).

Photosynthetic production

As with methanotrophic production, we sought to calculate true photosynthetic production i.e., the carbon that is assimilated into the algal biomass (and not immediately respired) and is therefore available for higher trophic levels. In our previous publication (Shelley et al. 2014) we estimated net ecosystem production (NEP), directly from the oxygen evolution in the light part of the incubation, and then compared NEP to our direct measurements of net

methanotrophy. However, NEP includes respiration from many non-photosynthetic components of the biofilm (including methanotrophs) and can be a negative number. Although it cannot be measured directly, an alternative is to estimate net photosynthetic production (NPP) from gross photosynthesis (GPP) by multiplying the latter by published estimates of carbon conversion efficiencies $\sim 50\%$ for algal photosynthesis (Cannell and Thornley 2000). Our measurements of GPP were scaled using a photosynthesis-irradiance (PI) curve constructed with either gravels from the River Lambourn (survey) (Shelley et al. 2014) or the substratum from the artificial channels (*see* Supporting Information S3). A scaling factor was calculated using the photosynthetically active radiation (PAR) at the riverbed as measured each field site, relative to that in the laboratory incubation chambers. After adjustment for riverbed irradiance, the rates of net photosynthetic production were calculated as follows:

$$\text{NPP} \left(\text{nmol cm}^{-2} \text{d}^{-1} \right) = \text{GPP} \times V \times \text{CCE} \times h \quad (3)$$

Whereby gross photosynthesis (GPP, in $\text{nmol g}^{-1} \text{h}^{-1}$) was multiplied by (V) the volume taken up by 1 g of sediment (0.95 cm^3), then the carbon conversion efficiency (CCE) of photosynthesis (0.5), and the number of hours of sunlight at 51.5°N on each sampling date (h). For simplicity, riverbed irradiance was assumed to be constant (at measured intensity) throughout the hours of daylight (*see* Discussion for modelled error estimates).

Statistical analyses

We used linear mixed effects models to determine whether there were differences between the measured variables (for example methane concentration, methane oxidation) in the unshaded and shaded gravels. For the survey data, river was fitted as a random effect and shading as a fixed effect. For the seasonal study and the channels, date was fitted as a repeat measure, random effect. We used the Akaike Information Criterion (AIC) to compare the fit of models (e.g., random intercept and slope vs. simpler, intercept only models).

For the channels, only the final 3 months of data were used, as colonization was still occurring during the first 2 months (assessed from monthly measures of methane oxidation). Linear mixed effects models were used instead of performing t -tests on mean values, or using the entire dataset, in order to retain all the variance in our analyses without inflating the degrees of freedom. A log-likelihood test was used to determine the level of significance of our model output. All statistical analyses were performed in R using the LME4 package (Bates et al. 2015).

Results

Site characteristics and methane concentrations

The survey sites were all typical chalk streams, with high pH (7.20–8.75) and $\sum\text{DIC}$ (2.5–4.6 mmol L^{-1}), clear water

and clean gravel beds (Table 1). All sites were supersaturated with both carbon dioxide and methane relative to the atmosphere. The concentration of methane ranged from 38 $\text{nmol CH}_4 \text{ L}^{-1}$ in the Misbourne to 224 $\text{nmol CH}_4 \text{ L}^{-1}$ in the Piddle (Table 1; Fig. 1a) and, on average across all 15 rivers, it was $7 (\pm \text{SE } 2.7) \text{ nmol L}^{-1}$ higher in shaded than in the unshaded areas ($X^2_{(1)} = 6.12$, $p = 0.01$). The concentration of carbon dioxide ranged from 77 to 513 $\mu\text{mol CO}_2 \text{ L}^{-1}$ and did not differ between the shaded and unshaded areas of the rivers ($X^2_{(1)} = 0.099$, $p = 0.75$). Geographical variation in the concentration of methane in August was greater than the entire annual range at the seasonal study site (Bere Stream: 36–192 $\text{nmol CH}_4 \text{ L}^{-1}$, Fig. 1b), where we measured a typical summer peak known to be associated with increased fine sediment deposition (Sanders et al. 2007; Shelley et al. 2015). The summer peak in methane concentration was more pronounced in the shaded area than the unshaded area on the Bere Stream (Fig. 1b) and, over the entire year, the methane concentration was slightly higher (mean difference = $7 \pm \text{SE } 1.8 \text{ nmol CH}_4 \text{ L}^{-1}$) in the shade ($X^2_{(1)} = 14.6$, $p < 0.001$). The concentration of nitrate ranged from 195 $\mu\text{mol L}^{-1}$ to 1716 $\mu\text{mol L}^{-1}$ (rivers Darent and Stort, respectively) and of SRP from 0.2 $\mu\text{mol L}^{-1}$ to 82.6 $\mu\text{mol L}^{-1}$ (rivers Allen and Stort, respectively, Table 1), both driven by the prevailing land use in the catchment and the proximity of sewage treatment plant outlets.

The manipulations applied to the artificial channels were effective. The low, medium, and high methane treatments averaged 9, 47, and 230 $\text{nmol CH}_4 \text{ L}^{-1}$, respectively (Fig. 1) and, despite small weekly variations in the concentrations of methane, they remained statistically different throughout ($X^2_{(1)} = 9.41$, $p = 0.002$) our experiment. The shading treatment had no effect on the concentration of methane ($X^2_{(1)} = 0.74$, $p = 0.39$) and the range of concentrations encompassed those measured in the river survey (Fig. 1).

Methanotrophic capacity

Biofilms on all gravels from both the river survey and the artificial channels could oxidize methane. Across the 15 rivers, the capacity for methane oxidation was 78% higher in gravels from shaded areas than in those from unshaded areas (Fig. 2a, $X^2_{(1)} = 7.04$, $p = 0.01$) with means of $0.57 (\pm \text{SE } 0.041) \text{ nmol CH}_4 \text{ g}^{-1} \text{h}^{-1}$ and $0.32 (\pm \text{SE } 0.019) \text{ nmol CH}_4 \text{ g}^{-1} \text{h}^{-1}$, respectively. The greatest capacity for methane oxidation was measured in gravels taken from the shaded area of the Piddle (Fig. 2a, $1.74 \text{ nmol g}^{-1} \text{h}^{-1}$), which was also the river with the highest methane concentration. However, across these 15 rivers, there was no relationship between ambient methane concentration and the capacity to oxidize methane ($X^2_{(1)} = 0.21$, $p = 0.65$).

In the seasonal study of the Bere Stream, there was no difference between the methanotrophic capacity in the gravels from the shaded and unshaded areas when data from all

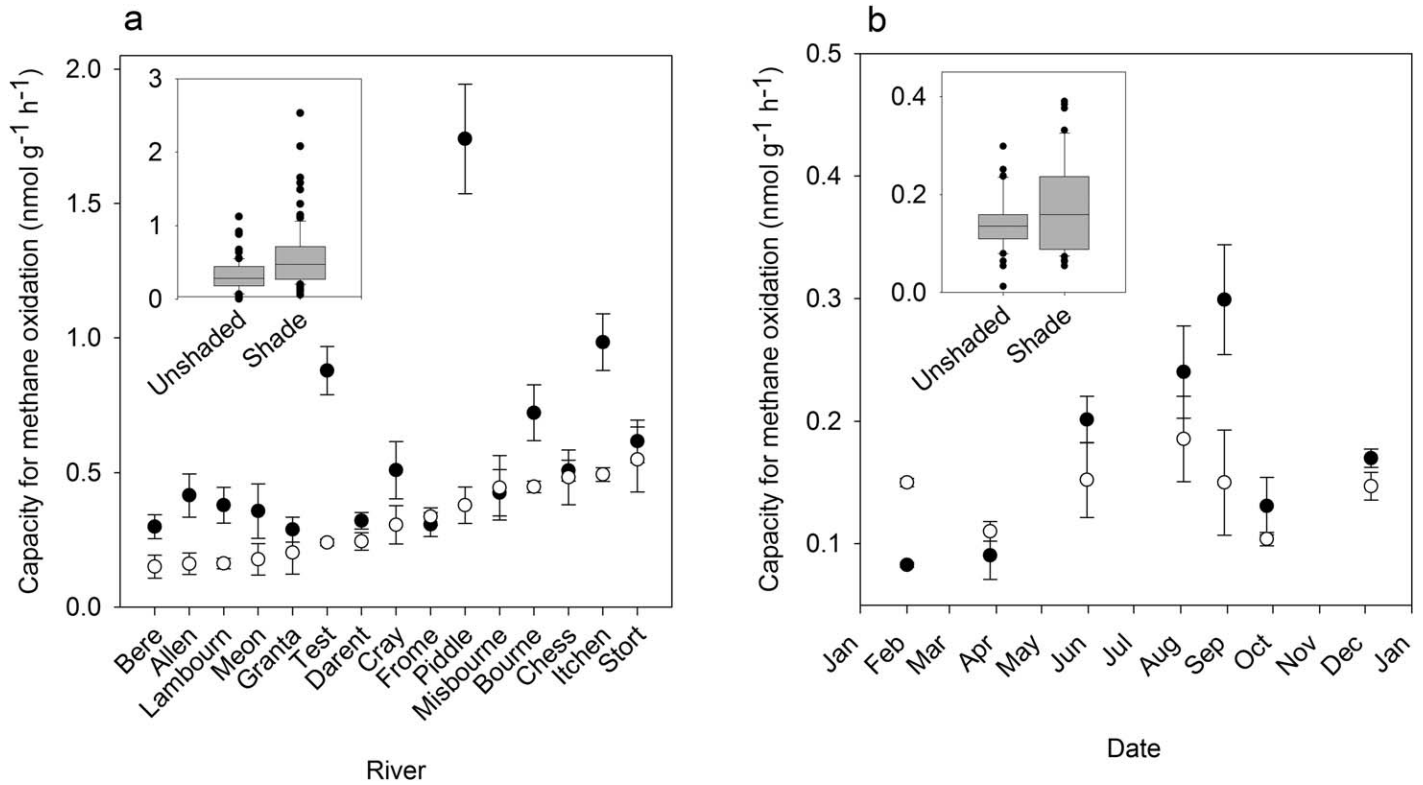


Fig. 2. Measured capacities (mean \pm SE) for methane oxidation at 100 nmol CH₄ L⁻¹ in the riverbed gravels from (a) unshaded (open circles) and shaded (filled circles) areas of the 15 rivers, and (b) the Bere Stream over the annual cycle. Insets are boxplots of the full dataset for unshaded and shaded gravels, irrespective of river (a) or season (b).

Table 2. Mean (\pm SE, $n = 5$) capacity for methane oxidation and estimates of methanotrophic production in the gravels from the six different treatments in the experimental channels.

Methane treatment	Capacity for methane oxidation (nmol CH ₄ g ⁻¹ h ⁻¹)		Methanotrophic production (nmol C cm ⁻² d ⁻¹)	
	Unshaded	Shaded	Unshaded	Shaded
Low	0.03 \pm 0.002	0.04 \pm 0.003	0.13 \pm 0.009	0.19 \pm 0.015
Medium	0.03 \pm 0.002	0.04 \pm 0.004	0.83 \pm 0.056	1.05 \pm 0.120
High	0.06 \pm 0.004	0.07 \pm 0.005	7.79 \pm 0.527	8.55 \pm 0.630

seven dates were included in the model ($X^2_{(1)} = 1.52$, $p = 0.22$). However, over the summer period (May–September), when shading was most intense, the capacity for methane oxidation was higher in the gravels from the shade (Fig. 2b, $X^2_{(1)} = 8.78$, $p = 0.003$). Moreover, there was a significant relationship between the ambient methane concentration and the capacity for methane oxidation ($X^2_{(1)} = 10.9$, $p = 0.001$), with the highest capacity in September (0.29 nmol CH₄ g⁻¹ h⁻¹) when methane was near its annual maximum, and lowest in February (0.08 nmol CH₄ g⁻¹ h⁻¹, Fig. 2b) when the methane concentration was at the winter baseline (Fig. 1b). When analyzed separately, there was no

relationship between methane concentration and the capacity for methane oxidation in the unshaded area, although there was in the shaded area.

In the artificial channels, there was a strong positive effect of methane concentration ($X^2_{(1)} = 60.9$, $p < 0.001$) and shading ($X^2_{(1)} = 8.39$, $p = 0.004$) on the capacity for methane oxidation in the gravels. When all incubated with the same initial methane concentration (100 nmol L⁻¹), the gravels' measured capacities for methane oxidation ranged from 0.03 nmol CH₄ g⁻¹ h⁻¹ in the unshaded, low methane treatment, to 0.07 nmol CH₄ g⁻¹ h⁻¹ in the shaded, high methane treatment (Table 2).

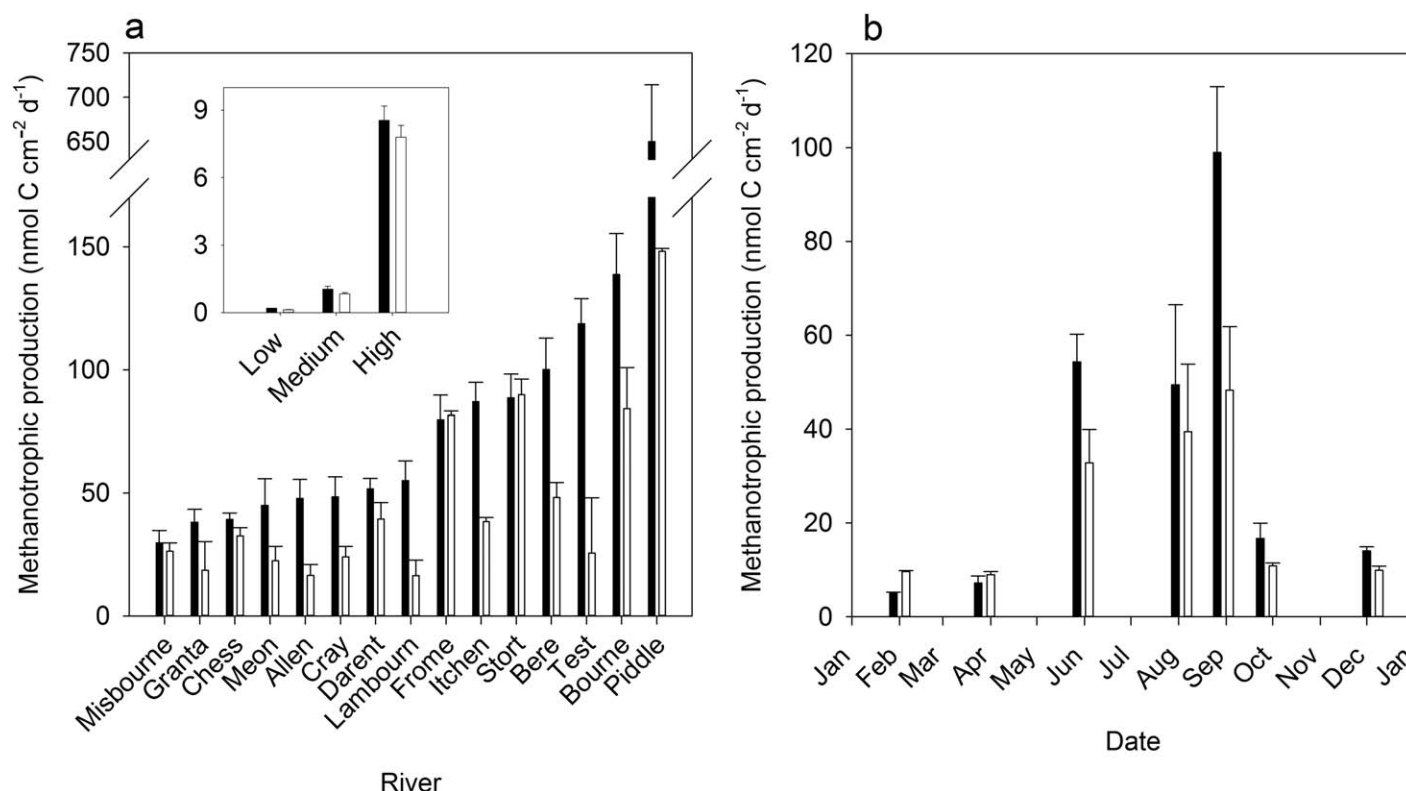


Fig. 3. Estimated daily carbon assimilation via methanotrophy (means \pm SE) in unshaded (gray bars) and shaded (black bars) areas of **(a)** the riverbeds of the 15 rivers **(a inset, the artificial channels at high, medium and low methane concentration)** and **(b)** the Bere Stream. Estimates use the measured capacity for methane oxidation normalized by the ambient methane concentration and scaled over the top 15 cm of the riverbed (or 5 cm for the artificial channels, their maximum depth).

Methanotrophic production

Methanotrophic production was estimated over the top 15 cm of the riverbed, as this is a conservative estimate of the likely vertical extent of methanotrophy in the bed of chalk streams (Pretty et al. 2006; Trimmer et al. 2010; Shelley et al. 2014). Across the 15 rivers, methanotrophic production ranged from 16 nmol C cm⁻² d⁻¹, in the unshaded areas of the Lambourn and the Allen, up to 650 nmol C cm⁻² d⁻¹, in the shaded area of the Piddle (Fig. 3a). On average, there were 60 nmol C cm⁻² d⁻¹ more carbon incorporated via methanotrophy in the shaded areas than in the unshaded areas of the riverbeds ($X^2_{(1)} = 32.4$, $p < 0.001$). In the artificial channels, methanotrophic production was much lower due to their restricted sediment depth (5 cm) and lower capacity for methane oxidation (Fig. 3a). Here, methanotrophy incorporated 0.16 nmol C cm⁻² d⁻¹, 0.95 nmol C cm⁻² d⁻¹, and 8.17 nmol C cm⁻² d⁻¹ in the low, medium and high methane treatments, respectively, and there was no difference between the shaded and unshaded treatments (Table 2) ($X^2_{(1)} = 1.60$, $p = 0.21$).

In the seasonal study on the Bere Stream, methanotrophic production varied seasonally, peaking at 99 nmol C cm⁻² d⁻¹ in the shaded area in late August and, by February

(when the concentration of methane had been low all winter), this was only 5 nmol C cm⁻² d⁻¹ (Fig. 3b). Over the year, methanotrophic production was greater in the shaded area of the riverbed compared to that in the unshaded area ($X^2_{(1)} = 6.48$, $p = 0.01$) and this was heavily driven by the large differences in the summer, when shading and methane concentration were maximal (Fig. 3b).

Light and photosynthetic production

In the 15 rivers, biofilms on gravels from the shaded areas received 89% less light than those from the unshaded areas (Fig. 4a; Table 1). The half-saturation constant for the photosynthesis-irradiance curve created from riverbed gravels was 39 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. NPP was marginally greater in unshaded areas compared to shaded areas ($X^2_{(1)} = 4.01$, $p = 0.045$), ranging from 2.7–35.7 $\mu\text{mol C cm}^{-2} \text{d}^{-1}$ in the open to 0.3–29.0 $\mu\text{mol C cm}^{-2} \text{d}^{-1}$ in the shade (Fig. 4b).

In the Bere Stream, riparian trees were in leaf from May to late September, resulting in a 98.5% reduction in light reaching the riverbed in the shaded area in August. The mean summer (derived from May, August, and September samples) reduction in light at the riverbed in shaded sections was 96% and, even in winter (despite the trees shedding

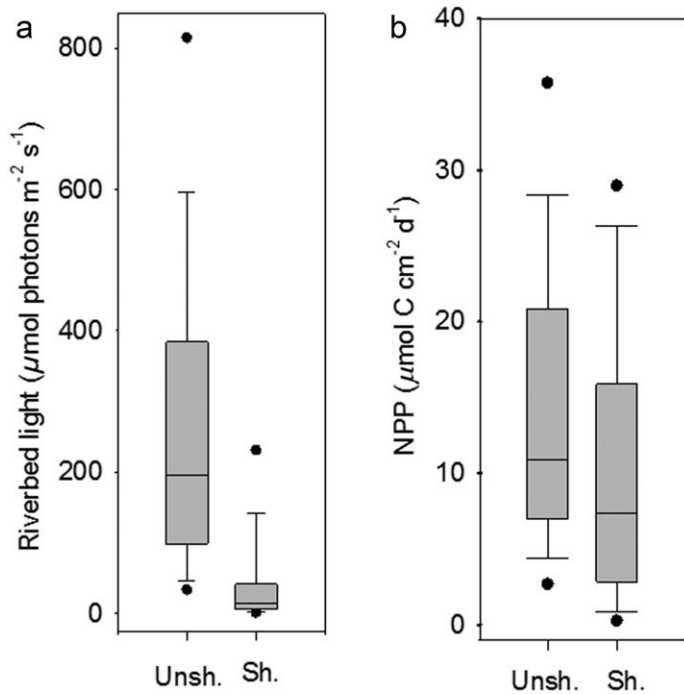


Fig. 4. Boxplots showing the difference in (a) riverbed light and (b) net photosynthetic production in unshaded and shaded areas of the 15 rivers in the survey. Boxes are formed from the interquartile range, mean values are shown as a horizontal line and the 10th and 90th percentiles are shown as whiskers with outliers as dots ($n = 15$).

their leaves), it was 72% (Fig. 5a). Not surprisingly given the latitude, PAR was greatly reduced in winter relative to summer, even in the unshaded area. In winter there was no difference in the NPP between unshaded and shaded sections ($X^2_{(1)} = 0.38$, $p = 0.54$) and it ranged from $0.2 \mu\text{mol C cm}^{-2} \text{d}^{-1}$ to $2.5 \mu\text{mol C cm}^{-2} \text{d}^{-1}$ (Fig. 5c). In summer NPP was $2.3 \mu\text{mol C cm}^{-2} \text{d}^{-1}$ higher from unshaded gravels ($X^2_{(1)} = 5.01$, $p = 0.03$; Fig. 5c).

In the experimental channels, the shading treatment reduced the light by 68% compared with the unshaded channels (Table 3). Despite this, NPP was not statistically different between light treatments ($X^2_{(1)} = 3.56$, $p = 0.06$).

The importance of methanotrophy for production

Methanotrophy contributed between 0.1% and 12.9% of the total carbon incorporated into biomass (via net photosynthesis and methanotrophy) across the whole spatial survey, between 0.1% and 2.0% in the artificial channels, and between 0.5% and 3.7% in the seasonal study of the Bere Stream (Fig. 6a). There was a positive correlation between ambient methane concentration and the importance of methanotrophy to total production in both unshaded and shaded sections (Fig. 6a) and this was retained throughout the annual cycle (Fig. 6b). The linear relationship between methane concentration and the importance of methanotrophic production in the gravels was 5.1 times steeper in shaded vs. unshaded sections (Fig. 6a) across 15 rivers in

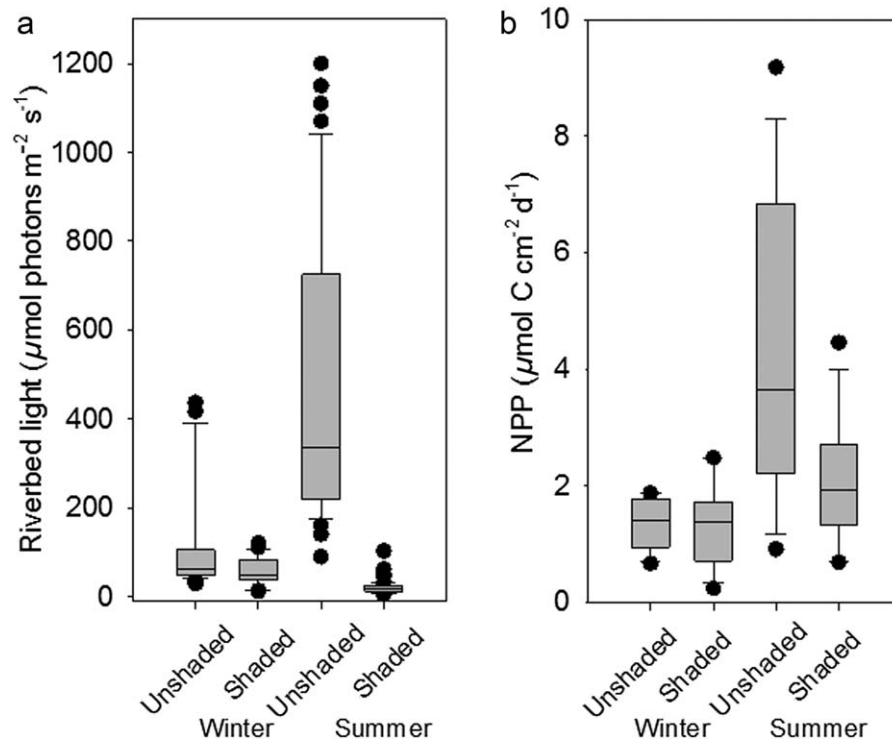


Fig. 5. Boxplots (see Fig. 4 for details) showing the difference in (a) riverbed light and (b) NPP in unshaded and shaded areas in summer (May–September) and winter (December–March) in the Bere Stream.

Table 3. Light and photosynthetic production (means \pm SE, $n = 15$) from the experimental channels.

Light treatment	Light ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	Net photosynthetic production ($\mu\text{mol C cm}^{-2} \text{ d}^{-1}$)
Unshaded	268 ± 18.9	1.31 ± 0.186
Shaded	86 ± 6.6	0.87 ± 0.236

summer. Within the seasonal study data, this relationship was 4.7 times steeper in the shade (Fig. 6b). The greater importance of methanotrophy in the shade is clear across all three components of our study (Fig. 6c). Points lying above the 1 : 1 line are rivers or artificial channels where methanotrophy accounted for a greater percentage of total production in the shade. This is the case in all but one of the rivers, in all three methane treatments in the artificial channels, and in all of the summer months in the seasonal study (Fig. 6c). The seasonal study shows that the difference in the relative importance of methanotrophic production is greatest in the summer (Fig. 6d) when it is approximately twice as important in the shade as in the open.

Discussion

We have shown that shading and methane concentration do affect both the capacity for methane oxidation and the contribution of methanotrophy relative to photosynthesis in rivers. By using three different yet complementary approaches, we were able to test our hypotheses fully against a backdrop of natural variation across 15 different lowland rivers, a seasonal cycle in one river, and in a more controlled setting in a suite of 30 experimental channels.

The seasonal and geographical variation in methane concentration was comparable to that previously published for other groundwater-fed streams (Jones and Mulholland 1998; Sanders et al. 2007; Shelley et al. 2014) and is probably a function of variation in catchment and in-stream methanogenesis (Sanders et al. 2007). The strong kinetic response of methanotrophy to methane concentrations in riverbed gravels has been described previously (Shelley et al. 2014; Trimmer et al. 2015), although in this study we have also shown a changing capacity for methane oxidation in response to methane concentration, both over an annual cycle and in an artificial channel experiment. This has not previously been demonstrated in riverbed gravels and implies active growth in the methanotrophic assemblage, over short time-scales (~ 2 months), that maximizes the use of the changing substrate availability as has been shown in sediment and soil core incubation studies (Amaral and Knowles 1995; Kightley et al. 1995).

In these riverbed sediments, as in many lakes and soils, methanotrophy is substrate limited and thus exhibits a linear increase in rate with increasing methane concentration (Bogner et al. 1997; Deutzmann et al. 2011; Shelley et al. 2014). The small, but significant difference in methane concentration between the shaded and unshaded sections of the rivers ($7 \text{ nmol CH}_4 \text{ L}^{-1}$ higher in the shade) will account for some of the additional methanotrophic production in the shade. However, we calculate that just 8% of the difference between methanotrophic production in the shaded and unshaded areas is due to ambient methane concentrations and the remaining 92% is due to true differences in the capacity for methane oxidation which may be due to light. Here, we measured methane concentration in the surface water as the gravel samples taken were from the surface. However, in future it might be beneficial to assess porewater methane concentration more closely in order to estimate more effectively the methanotrophic production in the sub-surface gravels. Whether the porewater methane is higher or lower than the surface water concentration, is probably a function of advective flow, fine sediment ingress and associated oxygen conditions.

The capacity for methane oxidation in the spatial survey (15 rivers) was not correlated with ambient methane concentration (in the overlying river water), but it did differ with shading (Fig. 2a). Methane oxidation capacity is generally correlated positively with methanotrophic abundance (Sundh et al. 2005; e.g., Deutzmann et al. 2011) and so it is likely that our measures of higher methanotrophic capacity in shaded areas reflected a difference in the abundance of methanotrophs. Why shading should have this effect requires explanation. We have already ruled out growth under marginally higher methane concentrations in the shade (see above). Furthermore, the increase in methanotrophic capacity in the shade was also found under the much more controlled conditions of our experiment, suggesting that this was indeed a real response to reduced light. Whether the underlying mechanism for this response to light is direct or indirect, or a combination of the two, requires further analysis of our results and the literature.

Methanotrophic bacteria reside in the epilithic biofilm, competing for space with algae and other microbial species, and it may be that in the shade, where algae (especially large cells; Hill et al. 2011) are less abundant, there is less competition for space. Further, photosynthetic organisms are likely to influence the concentrations of oxygen, labile carbon and nutrients within the biofilm, thus altering the environment for the methanotrophs and possibly affecting their diversity and community structure (Lyautey et al. 2005; Ylla et al. 2009). It is not possible to rule out some influence of these indirect drivers on the riverbed sediments.

It is possible that light directly inhibits methane oxidation in unshaded riverbeds. Photo-inhibition in ammonia-oxidizing bacteria, which are functionally similar to

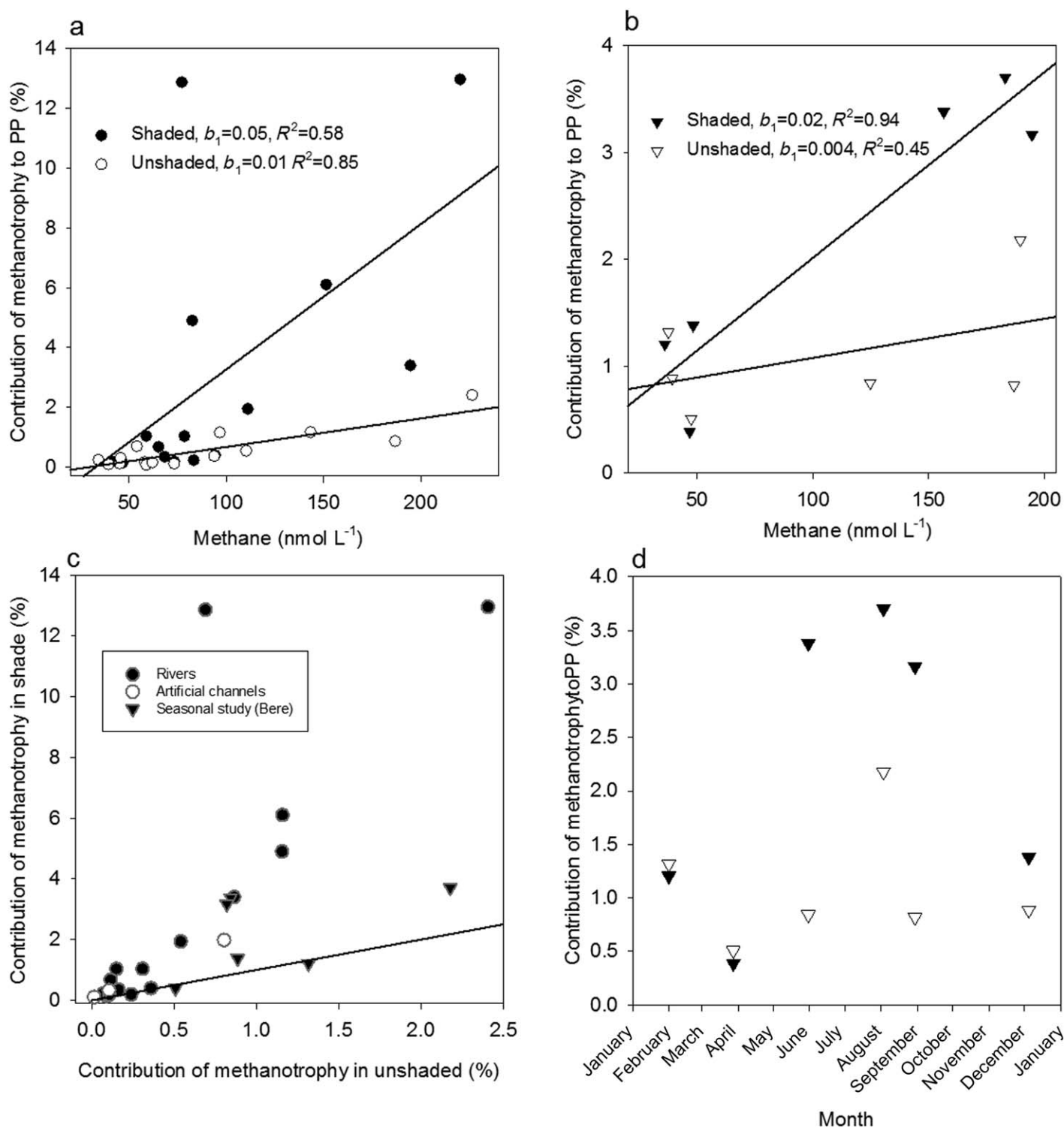


Fig. 6. Methanotrophic production as a percentage of total measured production ($(\text{MO}/(\text{MO} + \text{NPP})) \times 100$) in **(a)** the 15 rivers in August as a function of methane concentration across unshaded (unfilled circles) and shaded (filled circles) areas; and **(b)** as a function of methane concentration in the Bere Stream ($n = 6$ sampling occasions, with linear regression lines fitted); **(c)** percentage methanotrophy in unshaded areas plotted against that in the shaded areas in the 15 rivers (filled circles), artificial channels (open circles) and over an annual cycle (six occasions) in the Bere Stream (filled triangles), 1 : 1 ratio line added; **(d)** percentage methanotrophy in unshaded (open triangles) and shaded (filled triangles) areas in the Bere Stream plotted against date.

methanotrophs (O'Neill and Wilkinson 1977; Ward 1987), has been widely reported (Horrigan and Springer 1990; Guerrero and Jones 1996), and although this has been proposed for methane oxidation in lake water (Murase and Sugimoto 2005; e.g., Tang et al. 2014), it has never been shown in sediments. However, we have previously shown a higher capacity for methane oxidation on the undersides as compared to the tops of cobbles (Trimmer et al. 2009) and, while this may well be due to competition for space, it could also be that methanotrophs are directly sensitive to light. A similar distributional pattern on cobbles has been found for ammonia oxidizers in a longitudinal survey downstream of a sewage treatment works and was also attributed to a combination of these two factors (Ribot et al. 2012). If methanotrophs are photo-sensitive then we would either expect reduced functioning during daylight hours (short-term photo-inhibition), or a reduced population density in well-illuminated areas of riverbed (long-term population change). Given that all of our incubations were performed in the dark, and we still measured slower methanotrophy in gravels from unshaded areas of the riverbed, we reject the hypothesis that methanotrophs display short-term photo-inhibition, and instead we propose that either the abundance or the cell specific activity of methanotrophs is lower in gravels that are exposed to higher light conditions. Finally, it should be noted that beneath the surface layer, all sediments, regardless of surface irradiances, are shaded, and so any effect of light does not apply to the vast majority of the hyporheic zone. For this reason, we believe it was appropriate to up-scale our rates of methane oxidation, measured in the dark, to the top 15 cm of the hyporheic zone, but further studies should investigate the effect of light upon both surface and subsurface gravels to better understand this proposed phenomenon.

Our measurements of light and photosynthetic production show large variation across sites and seasons. Our PI curves show that the half-saturation constant (k_m) of the biofilm is $39.3 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (or $27.2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for the experimental channels, see electronic Supporting Information S3) meaning much of the light reaching the unshaded riverbed in summer (Figs. 4a, 5a), is above the saturation point and therefore has no additional impact on photosynthetic production (Figs. 4b, 5b). This explains why we do not see the extreme difference in riverbed light (Fig. 4a) propagated in the modelled rates of NPP (Fig. 4b). We used our spot measurements of riverbed PAR to estimate daily NPP from our laboratory measures because we did not have site-specific hourly riverbed PAR. For the purpose of error propagation, we crudely modelled changing riverbed irradiance and NPP across daylight hours and concluded that our approach (assuming constant irradiance equal to our spot measurement) resulted in between 12% and 31% (average 20%) over-estimation of NPP in the unshaded areas, and 18–38% (average 33%) in the shaded areas. These adjustments would make methanotrophy more important relative

to NPP, especially in the shaded areas, and would therefore make no difference to the overall outcome of our study.

Furthermore, shading from aquatic macrophytes can significantly reduce light, and therefore NPP [covering up to 80% of the riverbed in summer (Cotton et al. 2006; Trimmer et al. 2010; Shelley et al. 2014)] so in this study, we avoided macrophyte beds and only sampled from areas of the riverbed which were unshaded by riparian vegetation. Thus, our estimates of the importance of methanotrophy in the context of photosynthesis are conservative, and only apply to un-vegetated areas of riverbed.

Our overall estimates of the relative importance of methanotrophy show that it accounts for up to 13% of total production in the summer months, in line with our previous estimate of 11% in the Lambourn (Shelley et al. 2014). There are consistent patterns with methane concentration and light availability which can be seen across rivers, over an annual cycle, and in artificial channels (Fig. 6). Importantly, the measured reduction in methanotrophy in unshaded riverbed sediments is greater than its impact on photosynthesis. Thus, it is changes in methanotrophy which drive the difference in carbon assimilation between shaded and unshaded reaches, not changes in photosynthesis. This leads us to conclude that the reduced significance of methane-derived carbon in riverbed gravels in unshaded areas is most likely due to a combination of direct and indirect mechanisms acting upon methanotrophy, but the reduction of NPP in shaded areas enhances this reduction. This is an important finding when assessing the likely role of methane as a carbon source for higher trophic levels as it shows that the prevailing light conditions may affect the assimilation of methane into the biofilm.

Our results suggest that, where there are schemes to increase riverbed shading, generally driven by concerns over water temperature or algal blooms, they may also enhance riverbed methanotrophy and alter the balance of basal resources contributing to river food webs. Our methods for adjusting laboratory measurements for ambient light and methane concentrations, in order to model the balance of autochthony in sediments, could be applied to other benthic environments, such as wetlands, lakes, or estuaries with relative ease. To explore further the dynamics between riverbed irradiance and methane oxidation we propose using ^{13}C -labelled methane to measure the oxidation of methane to carbon dioxide in situ, under a range of natural light conditions. This could be performed using a benthic chamber approach (Hickey 1988) or by the tracer push-pull technique which has been used to great effect for quantifying nitrogen cycling (Lansdown et al. 2014). Expanding our approach beyond chalk rivers to other river types, particularly large, turbid rivers with high pelagic methanotrophic potentials, silty substrates, and shallow light penetration, may reveal a significant contribution from methanotrophic production to food webs to be a widespread phenomenon.

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Conflict of Interest

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